Regulation of flavin biosynthesis in the methylotrophic yeast Hansenula polymorpha

Brooke, Anthony G.; Dijkhuizen, Lubbert; Harder, Wim

Published in:
Archives of Microbiology

DOI:
10.1007/BF00413028

Arch Microbiol (1986) 145:62–70

Archives of
Microbiology

Regulation of flavin biosynthesis in the methylotrophic yeast *Hansenula polymorpha*

Anthony G. Brooke, Lubbert Dijkhuizen, and Wim Harder

Department of Microbiology, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands

Abstract. Under various conditions of growth of the methylotrophic yeast *Hansenula polymorpha*, a tight correlation was observed between the levels of flavin adenine dinucleotide (FAD)-containing alcohol oxidase, and the levels of intracellularly bound FAD and flavin biosynthetic enzymes. Adaptation of the organism to changes in the physiological requirement for FAD was by adjustment of the levels of the enzymes catalyzing the last three steps in flavin biosynthesis, riboflavin synthetase, riboflavin kinase and flavin mononucleotide adenylyltransferase. The regulation of the synthesis of the latter enzymes in relation to that of alcohol oxidase synthesis was studied in experiments involving addition of glucose to cells of *H. polymorpha* growing on methanol in batch cultures or in carbon-limited continuous cultures. This resulted not only in selective inactivation of alcohol oxidase and release of FAD, as previously reported, but invariably also in repression/inactivation of the flavin biosynthetic enzymes. In further experiments involving addition of FAD to the same type of cultures it became clear that inactivation of the latter enzymes was not caused directly by glucose, but rather by free FAD that accumulated intracellularly. In these experiments no repression or inactivation of alcohol oxidase occurred and it is therefore concluded that the synthesis of this enzyme and the flavin biosynthetic enzymes is under separate control, the former by glucose (and possibly methanol) and the latter by intracellular levels of free FAD.

Key words: *Hansenula polymorpha* – Regulation – Flavin biosynthesis – Methylotrophy – Repression – Inactivation – Riboflavin – Flavin mononucleotide – Flavin adenine dinucleotide – Alcohol oxidase

Following the recent identification of the origin of the C_4 moiety involved in the formation of 6,7-dimethyl-8-ribityllumazine (Nielson et al. 1984; Neuberger and Bacher 1985), the biosynthetic pathway for the formation of flavin adenine dinucleotide (FAD) in microorganisms (Fig. 1) is now know in considerable detail (for reviews see Demain 1972; Plaut et al. 1974; Bacher et al. 1983). Earlier work with *Saccharomyces cerevisiae* has identified 6 genes, rib_1 to rib_5 and rib_7 coding for several of the enzymes involved in the pathway (Bacher et al. 1969; Oltmanns et al. 1969; Oltmanns 1971). However, the regulation of this pathway is poorly understood. Whilst investigating the kinetics of flavinogenesis in prototrophic *Escherichia coli* strains, Wilson and Pardee (1962) demonstrated that riboflavin (Rf) and riboflavin-5′-phosphate (FMN) did not suppress riboflavin synthesis and suggested that flavinogenesis in *E. coli* was regulated at the level of enzyme synthesis by a repression – derepression mechanism rather than at the level of existing enzyme activities. However, these experiments were hampered by the impermeability of cells to exogenous Rf and did not include measurements of enzyme activities. Veldkamp et al. (1966) concluded that Rf production by growing cells of *Arthrobacter globiformis* was virtually unaffected by cultural conditions, and always occurred at the maximal rate, whereas Bresler et al. (1972) and Bacher et al. (1973) using Rf-requiring mutants demonstrated that the synthesis of riboflavin synthetase in *Bacillus subtilus* was subject to regulation by repression exerted by riboflavin.

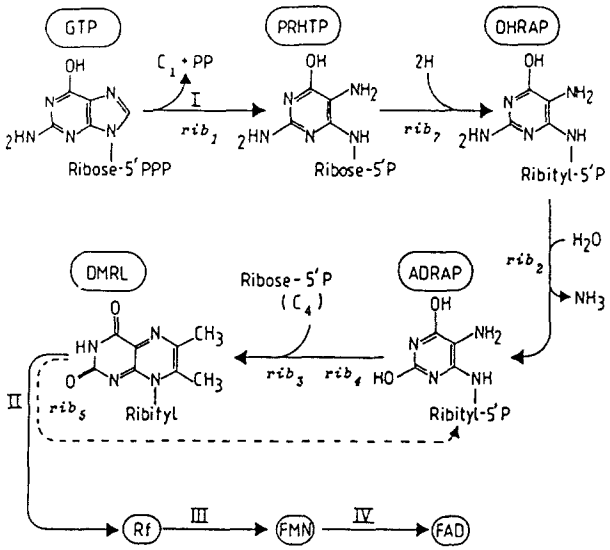

Fig. 1. Schematic representation of the pathway leading to the biosynthesis of FAD. The genes rib_1 to rib_5 and rib_7 identified in *Saccharomyces cerevisiae* are included in the reactions that the enzymes they code for catalyse: I, GTP cyclohydrolase; II, riboflavin synthetase; III, riboflavin kinase; IV, FMN adenylyltransferase.

Abbreviations. GTP, Guanosine-5′-triphosphate; *PRHTP*, phosphoribosyl-6-hydroxy-2,4,5-triaminopyrimidine-5′-phosphate; *DHRAP*, 2,5-diamino-6-hydroxy-4-ribitylaminopyrimidine-5′-phosphate; *ADRAP*, 5-amino-2,6-hydroxy-4-ribitylaminopyrimidine-5′-phosphate; *DMRL*, 6,7-dimethyl-8-ribityllumazine

Offprint requests to: A. G. Brooke

Abbreviations. FAD, Flavin adenine dinucleotide; FMN, riboflavin-5′-phosphate; flavin mononucleotide; Rf, riboflavin

It has been shown that in yeasts able to grow on methanol as sole carbon- and energy source, FAD-dependent alcohol oxidase catalyzes the first step of methanol oxidation (Tani et al. 1972a, b; Kato et al. 1976; Veenhuis et al. 1983). This enzyme contains 8 moles of FAD per mole of enzyme protein (Tani et al. 1972b; Kato et al. 1976) and dependent upon the growth conditions employed, considerable variation in the cellular levels of the enzyme is observed. During exponential growth of *Hansenula polymorpha* in batch cultures with excess glucose, synthesis of the enzyme is completely switched off, whereas at low dilution rates in methanol-limited continuous cultures it may constitute up to 30% of the soluble protein of the cell (Van Dijken et al. 1976). Therefore it seemed plausible that adaptive changes in the alcohol oxidase levels might be accompanied by changes in the intracellular flavin levels and their associated enzymes.

Eggeling et al. (1977) demonstrated that during growth of *Candida boidinii* on methanol, FMN adenylyltransferase was inducibly formed, and provided evidence that formation of this enzyme was correlated with the synthesis of alcohol oxidase apo-protein. Shimizu et al. (1977a, b) extended this study to *Kloeckera* sp. 2201 and included measurements of total intracellular and extracellular flavins. They proposed that the cellular flavin-level might be controlled by the amount of flavoprotein required physiologically. However, since intracellular flavins may be either bound to proteins or free within the cells (Wilson and Pardee 1962) it is important to distinguish between these two groups of flavins during investigations aimed at elucidating regulatory mechanisms, since marked changes in these pools may be associated with different growth responses.

This paper describes the results of an investigation in the methylotrophic yeast *H. polymorpha* into the activities of alcohol oxidase and flavinogenic enzymes in relation to changes in the intracellular flavin-pools, in particular the concentrations of free and bound FAD, both in batch and methanol-limited continuous cultures.

Materials and methods

Microorganism and cultivation. *Hansenula polymorpha* CBS4732 was used in all experiments. In batch culture studies the organism was grown at 37° C in 2.5 l Erlenmeyer flasks containing 500 ml of the mineral medium described by Van Dijken et al. (1975) supplemented with the appropriate carbon sources (0.5% w/v) except methanol (0.5% v/v). During continuous culture studies, the organism was grown in methanol-limited (sR = 0.5% v/v) chemostat cultures according to Van Dijken et al. (1976). It was assumed that the cells were in steady-state after at least 5 volume changes of the culture at the same absorbance. For transition experiments either glucose or FAD were added directly to both culture vessels and medium reservoirs to the final concentrations indicated in the individual experiments. At appropriate time intervals samples were removed from the cultures and used for the determination of intracellular/extracellular flavin levels or for the preparation of cell-free extracts and measurements of enzyme activities (see below).

Extraction and measurement of flavins. The flavins in cells of *H. polymorpha* were extracted using a modified version of the method described by Wilson and Pardee (1962). Cells were harvested, rapidly cooled to 4° C, washed, resuspended in 50 mM potassium phosphate buffer pH 7.0 and then centrifuged to produce pellets containing 5 – 10 mg protein. All subsequent steps were carried out at 4° C in the dark. The pellets were extracted with either 0.05% (v/v) toluene or 5.0% (w/v) trichloroacetic acid (TCA) for 1 h to remove free- (cellular-pool) flavins or total- (free plus TCA-extractable) flavins, respectively. Tri-potassium orthophosphate (0.15 ml of a 1 M solution) was subsequently added to the TCA-extracts to bring the pH to approximately 7.0. The extracted flavins were then removed from the cell-debris by a 10 min centrifugation step using an Amicon MPS-1 micropartition system (Amicon B.V., The Netherlands). The deproteinized microsolutes were stored at – 20° C in the dark for subsequent flavin analysis. Supernatant samples were filtered through 0.2 μm membrane filters (Schleicher and Schüll, FRG) prior to flavin measurements.

Reversed-phase high-performance liquid chromatography (HPLC) was used to separate and measure Rf, FMN and FAD as described by Light et al. (1980). Analytical HPLC separations were performed using an M-6000A solvent delivery system equipped with a Wisp 710B injector and an M441 dual wavelength absorbance detector (Waters Assoc., The Netherlands). The column used was a μBondapak C-18 column (Waters Assoc., 0.39 × 30 cm) connected in series with a guard column, with 35% methanol and 65% water (5 mM ammonium acetate, pH 6.0) as the mobile-phase. The absorbance of the flavins in the column effluent was monitored at 436 nm, and quantified using a Waters Model 730 Data Module in the external standard quantitation mode. Using this technique flavin concentrations down to 0.01 μmol/g protein were reproducibly measured.

Extract preparation and enzyme assays. The preparation of cell-free extracts was carried out by the passage of washed-cells twice through a French pressure cell at a pressure of 6,895 kN/m^2.

The estimation of alcohol oxidase activity (EC 1.1.3.13) was performed as described by Van Dijken et al. (1976) and was such that the reaction rate was proportional to the amount of extract added and linear for at least 3 min. All assays of flavin biosynthetic enzymes were carried out at 37° C in the dark and were such that the reaction rates were linear for at least 1 h. Riboflavin synthetase (EC 2.5.1.9) was assayed by a modified version of the procedure described by Plaut and Harvey (1971). The assay was performed in a 1.5 ml reaction mixture containing (final concentrations): 50 mM potassium phosphate buffer pH 7.0, 10 mM sodium hydrogen sulphite and 0.6 mM 6,7-dimethyl-8-ribityllumazine. The reaction was initiated by the addition of crude cell-free extract (1 – 2 mg of protein) and incubated for 60 min. At the start and end of the incubation period, 0.5 ml samples were withdrawn from the assay mixture and added to an equal volume of 10% trichloroacetic acid in a centrifuge-tube. The coagulated protein was removed by centrifugation and the absorbance of the clear supernatant measured at 405 and 470 nm, in a single-beam Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer. The amount of Rf produced was determined according to Plaut and Harvey (1971). Riboflavin kinase (EC 2.7.1.26) was assayed using a modified version of the method described by Eggeling et al. (1977). The assay was performed in a 1 ml reaction mixture containing (final concentrations): 75 mM Tris-HCl buffer pH 8.2, 0.1 mM riboflavin, 1.0 mM adenosine-triphosphate, 1.5 mM magnesium chloride and

Table 1 A, B. Activities of alcohol oxidase and flavin synthesizing enzymes (**A**) and flavin levels (**B**) of *Hansenula polymorpha* grown on various carbon sources in batch culture

A

Carbon source	Specific activities of			
	Alcohol oxidase (μmol O_2/min · mg protein)	Riboflavin synthetase	Riboflavin kinase	FMN adenylyl-transferase
		(nmol/h · mg protein)		
Methanol	1.60	4.57	3.52	5.25
Glycerol	1.29	3.65	2.61	4.60
Ethanol	0.01	1.67	1.39	1.72
Glucose	0.01	1.69	1.05	1.60

B

Carbon source	Flavin concentrations (μmol/g protein)								
	Intracellular						Supernatant		
	FAD		FMN		Rf		FAD	FMN	Rf
	free	bound	free	bound	free	bound			
Methanol	0.08	1.06	< 0.01	0.14	< 0.01	< 0.01	< 0.01	< 0.01	0.11
Glycerol	0.04	0.74	< 0.01	0.05	< 0.01	< 0.01	< 0.01	< 0.01	0.04
Ethanol	0.10	0.60	< 0.01	0.07	< 0.01	< 0.01	< 0.01	< 0.01	0.03
Glucose	0.10	0.41	< 0.01	0.07	< 0.01	< 0.01	< 0.01	< 0.01	0.03

10 mM potassium fluoride. The reaction was initiated by the addition of crude cell-free extract (1 – 2 mg of protein), incubated for 60 min and terminated with 0.5 ml 15% trichloroacetic acid. The FMN formed was determined by HPLC as described. The FMN adenylyltransferase (EC 2.7.7.2) was assayed essentially as described by de Luca (1963). The test solution contained in a total volume of 3.0 ml (final concentrations) 33 mM Tris-HCl buffer pH 7.5, 0.6 mM FMN, 0.6 mM adenosine triphosphate and 1.0 mM magnesium chloride. The reaction was initiated by the addition of crude cell-free extract (2 – 5 mg of protein), incubated for 60 min and terminated by boiling the mixture for 3 min. The formed FAD was subsequently determined by measurement of the rate of oxygen consumption in the oxidation of D-alanine by D-amino acid oxidase. The FAD-free apoenzyme was prepared by dialysis as described by Massey and Curti (1966). Oxygen consumption was measured using a Clark-type oxygen electrode (Biological Oxygen monitor, Yellow Springs Instrument Co, Ohio, USA) in a 4 ml reaction mixture at 37° C containing (final concentrations); 50 mM sodium pyrophosphate buffer pH 8.5, 80 μg apoamino acid oxidase, and FAD-containing solution. The reaction mixture was pre-incubated for 3 min, and the reaction started by the addition of 80 mM D-alanine. Assays in which the substrate for the reaction was omitted, or in which the reaction was terminated immediately following substrate addition served as reagent blanks. Specific activities are expressed as μmol oxygen consumed/min · mg protein except for the flavin enzymes where the activities are expressed as nmol flavin produced/h · mg protein.

Other methods. Protein concentrations in whole cells and cell-free extracts were determined using a modified version of the Folin-Ciocalteu method described by Lowry et al. (1951). Bovin serum albumin was used as standard. Growth of the organism was monitored by measuring the absorbance at 663 nm in a Vitatron 280 colorimeter (Vitatron, Dieren, The Netherlands), and dry weight values were determined by heating 25 ml aliquots of washed cells at 110° C to constant weight.

Chemicals. 6,7-Dimethyl-8-ribityllumazine was a generous gift from Prof. A. Bacher, Lehrstuhl für Organische Chemie und Biochemie, der Techn. Universität, München, and Dr. S. Shimizu, Kyoto University, Kyoto, Japan. All other chemicals were of analytical reagent-grade obtainable from commercial sources.

Results

Flavin and enzyme level changes during growth of Hansenula polymorpha on various carbon sources in batch cultures

The results of Table 1 A show that the activities of riboflavin synthetase, riboflavin kinase and FMN adenylyltransferase are tightly correlated with the activity of alcohol oxidase. Changes in these enzyme levels as a result of the growth substrate were reflected by the intracellular concentration of bound FAD, which increased 1.5 – 2-fold in methanol- and glycerol-grown cells compared with ethanol- and glucose-grown cells (Table 1 B). Apart from relatively high concentrations of bound FMN and extracellular Rf in methanol-grown cells, no significant changes in the levels of other flavins were observed. Interestingly, no free intracellular FMN nor extracellular FAD or FMN were detected. These results suggest that the flavinogenic enzyme activities

are tightly coupled to the cellular flavin requirement and that their regulatory control may be related to the control of alcohol oxidase synthesis. To investigate this in more detail, the activities of alcohol oxidase, the flavin biosynthetic enzymes and the flavin levels were followed during growth of *H. polymorpha* in batch culture on methanol.

Flavin and enzyme level changes during growth of Hansenula polymorpha on methanol in batch culture

When glucose-grown cells of *H. polymorpha* were transferred to methanol mineral medium, a lag-period of 5 h was observed before synthesis of alcohol oxidase and growth started. From this moment on a concomitant increase in the activities of the flavin-synthesizing enzymes was observed, the differential rates of synthesis being virtually identical throughout growth, suggesting that these enzymes are coordinately regulated (Fig. 2). In this experiment the most significant change in flavin levels was that of intracellularly bound FAD, which increased approximately 8-fold following transfer from glucose- to methanol growth (Table 2). It may be calculated that this increase in bound FAD corresponds almost exactly to the increase in alcohol oxidase-bound FAD during growth on methanol (see Discussion). This suggests that the organism possesses a very sensitive control system for the coordinate regulation of the last three enzymes involved in the flavin biosynthetic pathway which may be coupled to the intracellular FAD-requirement for alcohol oxidase holo-enzyme formation.

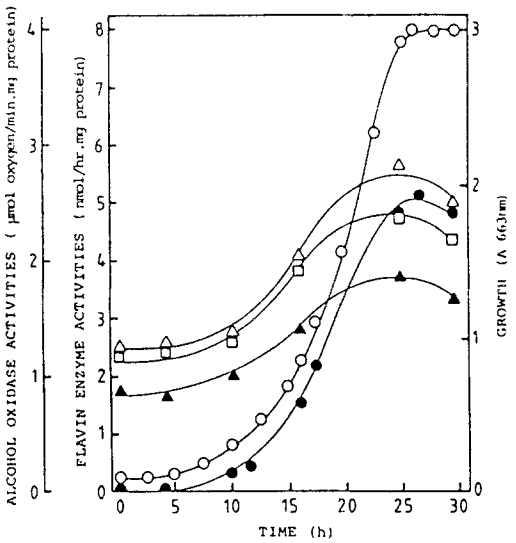

Fig. 2. Changes in the levels of alcohol oxidase and enzymes of flavin biosynthesis during growth of *Hansenula polymorpha* on methanol in batch culture. Cells were pre-grown to mid-exponential growth phase on glucose harvested, washed and used as the inoculum for growth on methanol. ○, Growth; specific activities of, ●, alcohol oxidase; △, FMN adenylyltransferase; ▲, riboflavin kinase; □, riboflavin synthetase

Effect of the addition of either glucose or FAD to cells of Hansenula polymorpha growing on methanol in batch cultures

Previous studies have demonstrated that in *H. polymorpha* the synthesis of alcohol oxidase is induced by the presence of methanol and sensitive to catabolite repression exerted by the metabolism of glucose (Van Dijken et al. 1975). In addition this enzyme is controlled at the level of existing activity via selective inactivation in the presence of glucose (Veenhuis et al. 1978; Bormann and Sahm 1978). Since our results indicate that the regulation of the last three enzymes responsible for FAD biosynthesis are tightly linked to that of alcohol oxidase, it became important to determine whether glucose exerted a similar effect on the flavin biosynthetic enzymes and thus on the flavin levels in this organism.

The results of such an investigation are shown in Fig. 3A and Table 3A. Immediately upon the addition of glucose to cells of *H. polymorpha* growing on methanol in batch culture, there was a sharp decrease in the specific activity of alcohol oxidase and a temporary slight decrease in the growth rate as the cells adapted to growth on glucose. Accompanying this change was a marked and simultaneous decrease in the specific activities of the flavin-synthesizing enzymes, which retained approximately 50% of their original activities after 4 h (Fig. 3A). A comparison of the flavin levels revealed that immediately following glucose addition to cells growing on methanol, the concentration of protein-bound FAD strongly decreased (Table 3A). In this phase free FAD accumulated intracellularly to a maximum concentration of 1.0 µmol/g protein and after 2 h FAD also began to accumulate extracellularly. There was no evidence to suggest any decomposition of FAD during this metabolic change since the concentration of free/bound intracellular- and extracellular FAD, expressed per gram of protein, always

Table 2. Changes in the intracellular and extracellular flavin levels during growth of *Hansenula polymorpha* on methanol in batch culture

Time (h)	Flavin concentrations (µmol/g protein)								
	Intracellular						Supernatant		
	FAD		FMN		Rf		FAD	FMN	Rf
	free	bound	free	bound	free	bound			
0	0.15	0.40	< 0.01	0.20	< 0.01	< 0.01	< 0.01	< 0.01	0.08
4	0.10	0.48	0.01	0.16	< 0.01	< 0.01	< 0.01	< 0.01	0.09
10	0.10	0.69	0.01	0.18	< 0.01	< 0.01	< 0.01	0.01	0.11
12	0.10	0.78	0.01	0.18	< 0.01	< 0.01	< 0.01	0.02	0.11
16	0.11	1.35	0.01	0.17	< 0.01	< 0.01	< 0.01	0.03	0.16
24	0.09	3.01	0.02	0.18	< 0.01	< 0.01	< 0.01	0.05	0.25
30	0.10	3.14	0.03	0.19	< 0.01	< 0.01	< 0.01	0.06	0.27

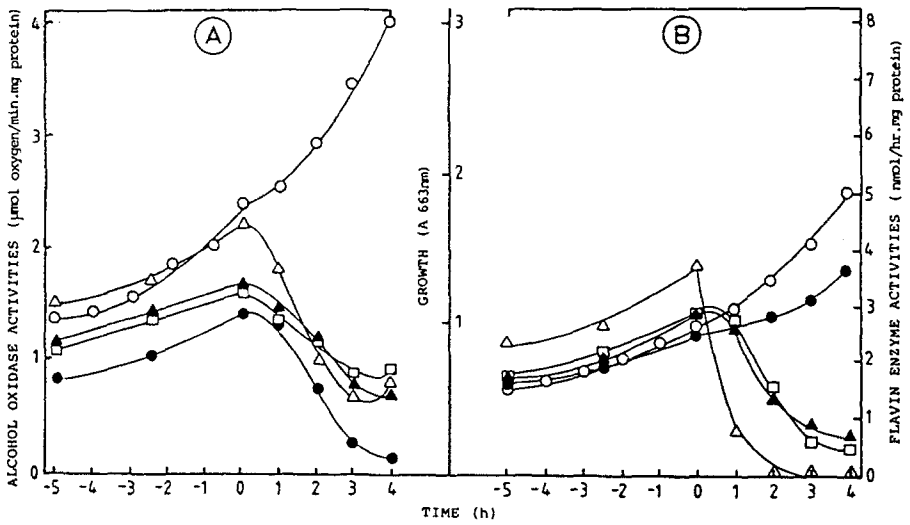

Fig. 3 A, B
Changes in the levels of alcohol oxidase and enzymes of flavin biosynthesis during growth of *Hansenula polymorpha* on methanol in batch culture following the addition of either glucose (0.5% w/v) or FAD (10 µM). Cells pregrown as described in the legend of Fig. 2 were grown to midexponential growth phase on methanol (represented by $T = 0$), at which point either glucose **A** or FAD **B** was added to the culture. Thereafter samples were removed every 30 min and monitored as described in Methods. *Symbols*: as in Fig. 2

Table 3. A Changes in the intracellular and extracellular flavin levels during growth of *Hansenula polymorpha* on methanol in batch culture following the addition of glucose (0.5% w/v)

Time (h)	Flavin concentrations (µmol/g protein)								
	Intracellular						Supernatant		
	FAD		FMN		Rf		FAD	FMN	Rf
	free	bound	free	bound	free	bound			
0	0.08	2.00	0.02	0.18	< 0.01	< 0.01	< 0.01	0.03	0.18
1	0.15	1.79	0.01	0.17	< 0.01	< 0.01	< 0.01	0.02	0.17
2	0.823	0.72	0.01	0.18	< 0.01	< 0.01	0.12	0.01	0.16
3	1.00	0.21	0.01	0.20	< 0.01	< 0.01	0.26	< 0.01	0.16
4	0.83	0.10	0.01	0.17	< 0.01	< 0.01	0.40	< 0.01	0.14

B Changes in the intracellular and extracellular flavin levels during growth of *Hansenula polymorpha* on methanol in batch culture following the addition of FAD (10 µm)

Time (h)	Flavin concentrations (µmol/g protein)								
	Intracellular						Supernatant		
	FAD		FMN		Rf		FAD	FMN	Rf
	free	bound	free	bound	free	bound			
0	0.07	1.70	0.02	0.17	< 0.01	< 0.01	47.62	0.03	0.16
1	2.68	1.77	0.02	0.18	< 0.01	< 0.01	37.40	0.03	0.15
2	5.07	1.96	0.02	0.18	< 0.01	< 0.01	27.97	0.03	0.17
3	7.00	2.17	0.02	0.17	< 0.01	< 0.01	22.38	0.03	0.18
4	8.63	2.59	0.02	0.18	< 0.01	< 0.01	18.45	0.03	0.17

approximated to the amount of free/bound FAD prior to the addition of glucose. On the basis of these results it might be concluded that the enzymes of the flavin-biosynthetic pathway in *H. polymorpha*, like alcohol oxidase, are very sensitive to control by glucose. It remained possible, however, that the intracellular pool of free FAD, rather than glucose itself, controlled the levels of the flavin biosynthetic enzymes. Clearly, control of the activities of these enzymes in *H. polymorpha* by FAD, the end-product of the flavin biosynthetic pathway and prosthetic group of alcohol oxidase, would be a highly efficient control mechanism.

This possibility was investigated by adding FAD (10 µM) to mid-exponential phase cells growing on methanol. The results of this experiment are shown in Fig. 3B and Table 3B. Immediately upon the addition of FAD the intracellular concentration of free FAD increased and this change was accompanied by an immediate and rapid decrease in the specific activities of riboflavin synthetase, riboflavin kinase and FMN adenylyltransferase. This response was most marked with the latter enzyme which retained only 7% of its original activity after 2 h. However, growth and the activity of alcohol oxidase remained

unaltered (Fig. 3B). In this experiment again no decomposition of FAD was observed. In control experiments in which the effect of addition of FAD to glycerol-grown cells was studied, essentially the same results were obtained. Moreover, accumulation of free intracellular Rf or FMN following the addition of these compounds to exponentially-growing cells on methanol did not elicit such a response (data not shown). These results show that the control of the levels of the flavin-synthesizing enzymes in *H. polymorpha* is different to that of alcohol oxidase and appears to be related to the intracellular concentration of free FAD.

Flavin and enzyme level changes during growth of Hansenula polymorpha on methanol in continuous cultures

In order to shed more light on the regulatory mechanisms controlling flavin biosynthesis, the relationship between the requirement for FAD, due to alcohol oxidase apo-protein formation, and the levels of flavin-synthesizing enzymes was investigated in methanol-limited continuous cultures. Figure 4 illustrates the effect of varying the dilution rate on enzyme synthesis in methanol-limited cells of *H. polymorpha*. In accordance with the observations of van Dijken et al. (1976) it can be seen that alcohol oxidase became derepressed with decreasing dilution rates, its specific activity increasing almost 2-fold from $D = 0.150\ h^{-1}$ to $D = 0.025\ h^{-1}$. Concomitantly the activities of the flavin-synthesizing enzymes and the level of intracellularly bound FAD (Table 4) increased approximately 2-fold over the same range of dilution rates. As shown above in the batch culture experiments, methanol-grown cells generally possess small quantities of intracellular free FAD/FMN, and FMN in the culture supernatants (Table 2). This, however, was not the case in the carbon-limited continuous cultures (Table 4). These results are consistent with the idea that the levels of the flavin enzymes are controlled in such a way that an increased cellular requirement for FAD, due to derepressed alcohol oxidase levels at the lower dilution rates, can be satisfied. In addition this control mechanism apparently ensures that flavin overproduction does not occur under these conditions.

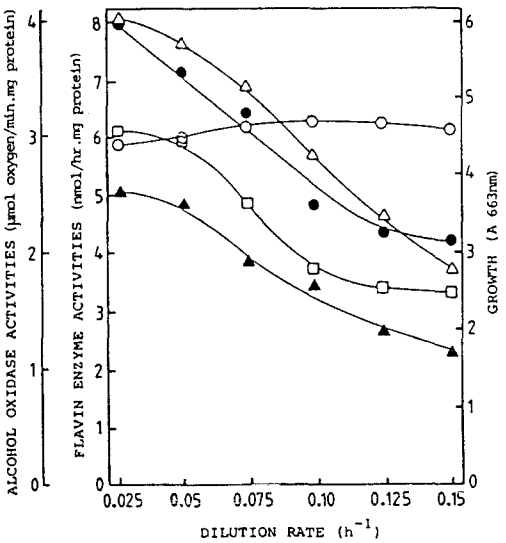

Fig. 4. Changes in the levels of alcohol oxidase and enzymes of flavin biosynthesis with dilution rate during growth of *Hansenula polymorpha* under methanol-limiting conditions in continuous cultures. *Symbols*: as in Fig. 2

The effect of addition of either glucose or FAD to cells of Hansenula polymorpha growing in methanol-limited continuous cultures

Immediately following the addition of glucose (0.5% w/v) to cells of *H. polymorpha* growing in continuous cultures on methanol ($D = 0.05\ h^{-1}$), the specific activity of alcohol oxidase rapidly decreased, retaining approximately 15% of its initial activity after 3.5 h (Fig. 5A). This change was paralleled by a similar decrease in the level of intracellularly bound FAD, and a corresponding increase in the intracellular free FAD concentration, which attained a value of approximately 3 µmol/g protein after 2 h before accumulating extracellularly (Table 5A). Over the same period the activities of the flavin-synthesizing enzymes decreased, this response being most pronounced with FMN adenylyltransferase which retained only 15% of its initial activity after 3.5 h, compared with 35% for riboflavin synthetase and riboflavin kinase. A comparison of the rate of decrease in the specific activities of the flavin-synthesizing enzymes to the rate due merely to cessation of enzyme synthesis coupled with diluting out of remaining activity over newly formed cells and wash-out of the cells, revealed that the decrease in activity was approximately 2-fold higher than could be accounted for by these processes. Hence these results demonstrate that, as is the case with alcohol oxidase,

Table 4. Changes in the intracellular and extracellular flavin levels during growth of *Hansenula polymorpha* on methanol in continuous culture

D (h^{-1})	Flavin concentrations (µmol/g protein)								
	Intracellular						Supernatant		
	FAD		FMN		Rf		FAD	FMN	Rf
	free	bound	free	bound	free	bound			
0.025	< 0.01	5.11	< 0.01	0.31	< 0.01	< 0.01	< 0.01	< 0.01	0.04
0.050	< 0.01	4.60	< 0.01	0.30	< 0.01	< 0.01	< 0.01	< 0.01	0.03
0.075	< 0.01	4.10	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.10
0.100	< 0.01	3.51	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.37
0.125	< 0.01	2.96	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.10
0.150	< 0.01	2.71	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.01

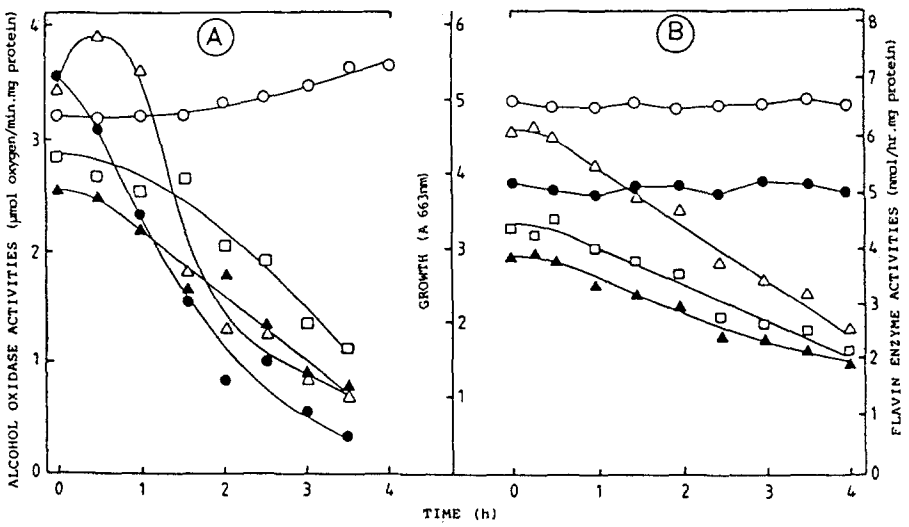

Fig. 5A, B
The effect of the addition of either glucose (0.5% w/v) (**A**) or FAD (10 µM) (**B**) to cells of *Hansenula polymorpha* growing in methanol-limited continuous cultures at dilution rates of 0.05 and 0.10 h^{-1} respectively. Prior to the transition the cells were growing in steady-state. At zero time, either glucose **A** or FAD **B** was added to both the culture vessel and medium reservoir. Samples of cells were then removed at 30 min intervals and monitored as described in Methods. *Symbols*: as in Fig. 2

Table 5. A Intracellular- and extracellular-flavin changes during transient-growth of *Hansenula polymorpha* following the addition of glucose (0.5% w/v) to methanol-grown cells in continuous culture ($D = 0.05\ h^{-1}$)

Time (min)	Flavin concentrations (µmol/g protein)								
	Intracellular						Supernatant		
	FAD		FMN		Rf		FAD	FMN	Rf
	free	bound	free	bound	free	bound			
0	< 0.01	4.53	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.40
15	0.03	4.47	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.46
30	0.22	4.26	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.37
45	0.69	3.83	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.39
60	0.90	3.56	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.39
90	2.06	2.27	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.40
120	3.00	1.33	< 0.01	< 0.01	< 0.01	< 0.01	0.30	< 0.01	0.39
150	3.12	1.46	< 0.01	< 0.01	< 0.01	< 0.01	0.99	< 0.01	0.41
180	3.15	1.07	< 0.01	< 0.01	< 0.01	< 0.01	1.34	< 0.01	0.37
210	4.00	0.96	< 0.01	< 0.01	< 0.01	< 0.01	1.07	< 0.01	0.41

B Intracellular- and extracellular-flavin changes during transient-growth of *Hansenula polymorpha* following the addition of FAD (10 µM) to methanol-grown cells in continuous culture ($D = 0.10\ h^{-1}$)

Time (min)	Flavin concentrations (µmol/g protein)								
	Intracellular						Supernatant		
	FAD		FMN		Rf		FAD	FMN	Rf
	free	bound	free	bound	free	bound			
0	< 0.01	3.32	< 0.01	< 0.01	< 0.01	< 0.01	9.52	< 0.01	0.40
15	< 0.01	3.24	< 0.01	< 0.01	< 0.01	< 0.01	9.15	< 0.01	0.37
30	0.05	3.45	< 0.01	< 0.01	< 0.01	< 0.01	9.34	< 0.01	0.42
45	0.11	3.12	< 0.01	< 0.01	< 0.01	< 0.01	9.58	< 0.01	0.39
60	0.18	3.25	< 0.01	< 0.01	< 0.01	< 0.01	9.34	< 0.01	0.36
90	0.37	3.23	< 0.01	< 0.01	< 0.01	< 0.01	9.46	< 0.01	0.37
120	0.47	3.20	< 0.01	< 0.01	< 0.01	< 0.01	9.60	< 0.01	0.40
150	0.54	3.26	< 0.01	< 0.01	< 0.01	< 0.01	9.41	< 0.01	0.40
180	0.67	3.41	< 0.01	< 0.01	< 0.01	< 0.01	9.53	< 0.01	0.30
210	0.74	3.39	< 0.01	< 0.01	< 0.01	< 0.01	9.53	< 0.01	0.39

the synthesis of riboflavin synthetase, riboflavin kinase and FMN adenylyltransferase is not only repressed, but that these enzymes also become inactivated under physiological conditions in which they are no longer required.

Following the addition of FAD (10 µM) to cells of *H. polymorpha* growing in continuous cultures on methanol ($D = 0.10\ h^{-1}$), free FAD was detectable intracellularly after 30 min (Table 5B). From this moment on there was a

rapid and simultaneous decrease in the activities of the flavin enzymes, which retained only 50% of their initial activities after 4 h (Fig. 5B). Since the decrease in these activities occurred at 2.5-fold the rate of cell wash-out, and the culture density remained unaltered over the same period, again this change cannot be explained on the basis of repression of enzyme synthesis alone, but indicates the involvement of an inactivation mechanism as well, most probably induced by enhanced levels of intracellular free FAD. Since the specific activity of alcohol oxidase remained unaltered over the same period, these results demonstrate furthermore that repression/inactivation of alcohol oxidase and the flavinogenic enzymes is under separate control, the former by glucose, and the latter by increasing concentrations of intracellular free FAD.

Discussion

In this paper a study has been made of the regulation of the synthesis of flavins in relation to the cellular-requirement in *Hansenula polymorpha* grown under various physiological conditions. This organism employs FAD-requiring alcohol oxidase during growth on methanol, an enzyme which is synthesized at greatly enhanced levels under certain cultural conditions (Van Dijken 1976; Eggeling and Sahm 1978). The results expressed in Table 1A, B demonstrate that high alcohol oxidase activities are correlated with similarly enhanced levels of intracellularly bound FAD. Furthermore, from the activities of alcohol oxidase and FAD levels at various stages during growth in batch culture on methanol (Table 2 and Fig. 2), and knowing that 1 mg of crystalline enzyme contains an activity of 11.0 μmol oxygen consumed/min · mg protein and 12.54 nmol FAD (Tani et al. 1972a; Kato et al. 1976), an estimation of the amount of alcohol oxidase-bound FAD may be made assuming that the relative amounts of other flavin enzymes remain unaltered. These theoretical values are 0, 0, 0.220, 0.315, 0.875, 2.736, 2.740 μmol/g protein at 0, 4, 10, 12, 16, 24 and 30 h respectively. Since the cells were pre-grown on glucose prior to growth on methanol, it may be assumed that the intitial concentration of bound FAD represents that required to satisfy the cellular requirement of FAD-containing enzymes other than alcohol oxidase, and should therefore be subtracted from the values in the table. Hence, a comparison of these values demonstrates clearly that in *H. polymorpha* there exists a very tight correlation between the cellular requirement for FAD (due to the formation of an active alcohol oxidase holoenzyme), and the rate of synthesis of this flavin. This organism thus offers an excellent model system to study the regulation of flavin biosynthesis in relation to the physiological requirement of the cells.

The results in Table 1 also show that *H. polymorpha*, like *Kloeckera* sp. 2201 (Shimizu et al. 1977a, b) can adapt to changes in the physiological requirement of the cells for flavins under different growth conditions by accurately adjusting the rates of synthesis of the last three enzymes of the flavin biosynthetic pathway. Furthermore, the differential rates of synthesis of these enzymes during growth on methanol (Figs. 2 and 4) indicate that they are coordinately induced by the presence of methanol, as previously suggested by Eggeling et al. (1977) for *Candida boidinii*.

Having seen that there is a strict control over the amount of FAD produced by cells of *H. polymorpha* in response to the cellular requirement for FAD, the question arises, how is this control achieved? In methylotrophic yeasts evidence for selective inactivation of alcohol oxidase and other enzymes involved in methanol metabolism has been described previously (Bormann and Sahm 1978; Veenhuis et al. 1978) and accounts for the observed loss in activity of alcohol oxidase when methanol-grown cells are exposed to glucose. Bruinenberg et al. (1982), using HPLC-techniques demonstrated that this decrease in alcohol oxidase activity was both due to a decrease in the amount of alcohol oxidase protein and to the dissociation of FAD from the enzyme. This observation prompted us to study whether glucose induced a similar inactivation of the flavin-synthesizing enzymes in methanol-grown cells of *H. polymorpha*. Immediately following the addition of glucose, not only was there a sharp decrease in the specific activity of alcohol oxidase, but also a simultaneous and corresponding decrease in the specific activities of riboflavin synthetase, riboflavin kinase and FMN adenylyltransferase (Figs. 3A and 5A). A comparison of the rates of decrease in these enzyme activities with wash-out kinetics in methanol-grown continuous cultures following the addition of glucose (Fig. 5A), revealed that the decrease in activities could not be explained by a catabolite repression mechanism only, but that inactivation, induced by the presence of glucose also occurred. However, a consideration of the intracellular free-/bound FAD concentration changes following glucose addition, and experiments in which the effects of extracellularly added FAD to methanol-grown cells (Figs. 3B, 5B) were studied, revealed that repression/inactivation of the flavin-synthesizing enzymes was not caused directly by the addition of glucose, but by the increasing concentration of free FAD which accumulated intracellularly during catabolite inactivation of alcohol oxidase by glucose or following its addition to the medium. These experiments demonstrated that alcohol oxidase is under separate control to the last three enzymes of the flavin biosynthetic pathway, since following FAD addition there was a sharp decrease in the specific activities of these enzymes, whereas the activity of alcohol oxidase remained unaltered. It should be noted that when the same concentration of FAD was included in the assays for flavin enzymes, this did not affect their activities. In these experiments it also became clear that in contrast to the situation observed in *E. coli* (Wilson and Pardee 1962), *H. polymorpha* is permeable to exogenously supplied flavins.

Hence in *H. polymorpha* the levels and activities of the flavin-synthesizing enzymes riboflavin synthetase, riboflavin kinase and FMN adenylyltransferase appear to be controlled by the level of free intracellular FAD. When the physiological demand for FAD increases i.e. at low dilution rates in methanol-limited continuous cultures, the synthesis of these enzymes increased because of a release of feedback repression to accommodate the increased requirement. On the other hand, when the physiological demand for FAD diminishes, the increasing intracellular concentration of free FAD causes feedback repression of the synthesis of these enzymes and additionally inactivation of the existing enzyme activities. To the knowledge of the authors this paper represents the first example of such a control system involved in flavin biosynthesis.

Acknowledgements. A. G. Brooke wishes to acknowledge receipt of an Overseas Postdoctoral Research Fellowship funded by S.E.R.C. England.

References

Bacher A, Baur R, Oltmanns O, Lingens F (1969) Biosynthesis of riboflavin in mutants of *Saccharomyces cerevisiae* accumulating 6OH, 2-4-5 triaminopyrimidine. FEBS Lett 5:316–318

Bacher A, Eggers U, Lingens F (1973) Genetic control of riboflavin synthetase in *Bacillus subtilis*. Arch Microbiol 89:73–77

Bacher A, LeVan Q, Keller PJ, Floss HG (1983) Biosynthesis of riboflavin. Incorporation of ^{13}C-labelled precursors into the xylene ring. J Biol Chem 258:13431–13437

Bormann C, Sahm H (1978) Degradation of microbodies in relation to activities of alcohol oxidase and catalase in *Candida boidinii*. Arch Microbiol 117:67–72

Bresler SE, Glazunov EA, Perumov DA (1972) Study on the riboflavin operon in *Bacillus subtilis*. IV. Regulation of biosynthesis of riboflavin synthetase. Study of riboflavin transport through the cell membrane. Genetika 8 (2):109–118

Bruinenberg PG, Veenhuis M, Van Dijken JP, Duine JA, Harder W (1982) A quantitative analysis of selective inactivation of peroxisomal enzymes in the yeast *Hansenula polymorpha* by high-performance liquid chromatography. FEMS Lett 15:45–50

Demain AL (1972) Riboflavin oversynthesis. Ann Rev Microbiol 26:369–388

Dijken JP van (1976) Oxidation of methanol by yeasts. Ph. D Thesis, University of Groningen, The Netherlands

Dijken JP van, Veenhuis M, Kreger-van Rij NJW, Harder W (1975) Microbodies in methanol assimilating yeasts. Arch Microbiol 102:41–44

Dijken JP van, Otto R, Harder W (1976) Growth of *Hansenula polymorpha* in a methanol-limited chemostat. Physiological responses due to the involvement of methanol oxidase as a key enzyme in methanol metabolism. Arch Microbiol 111:137–144

Eggeling L, Sahm H (1978) Derepression and partial insensitivity to carbon catabolite repression of methanol dissimilating enzymes in *Hansenula polymorpha*. Europ J Appl Microbiol Biotechnol 5:197–202

Eggeling L, Sahm H, Wagner F (1977) Induction of FMN adenylyltransferase in the methanol utilizing yeast *Candida boidinii*. FEMS Lett 1:205–211

Kato N, Omori Y, Ogata K (1976) Alcohol oxidases of *Kloeckera* sp. and *Hansenula polymorpha*. Catalytic properties and subunit structures. Eur J Biochem 64:341–350

Light DR, Walsh, C, Marletta MA (1980) Analytical and preparative high-performance liquid chromatography separation of flavin and flavin analog coenzymes. Anal Biochem 109:87–93

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Luca C de (1963) In: Colowick SP, Kaplan NO (eds) Methods in enzymology, vol VI. Academic Press, New York London, pp 342–345

Massey V, Curti B (1966) Conformational changes accompanying the binding of FAD to D-amino acid oxidase apoprotein. J Biol Chem 241:3417–3423

Neuberger G, Bacher A (1985) Biosynthesis of riboflavin. An aliphatic intermediate in the formation of 6,7-dimethyl-8-ribityllumazine from pentose phosphate. Biochem Biophys Res Commun 127: (1) 175–181

Nielsen P, Neuberger G, Floss HG, Bacher A (1984) Biosynthesis of riboflavin: Enzymatic formation of the xylene moiety from carbon ^{14}-labelled ribulose-5′phosphate. Biochem Biophys Res Commun 118 (3):814–820

Oltmanns O (1971) Riboflavinless mutants of *Saccharomyces cerevisiae*. The problem of the rib_6-gene. Mol Gen Genet III (3):300–302

Oltmanns O, Bacher A, Lingens F, Zimmermann FK (1969) Biochemical and Genetic classification of riboflavin deficient mutants of *Saccharomyces cerevisiae*. Mol Gen Genet 105 (4) 306–313

Plaut GWE, Harvey RA (1971) In: McCormick DM, Wright LD (eds) Methods in enzymology, vol XVIII, part B. Academic Press, New York London, pp 521–523

Plaut GWE, Smith CM, Alworth WL (1974) Biosynthesis of water-soluble vitamins. Ann Rev Biochem 43:899–922

Shimizu S, Ishida M, Tani Y, Ogata K (1977a) Flavin changes of *Kloeckera* sp. No. 2201 during adaptation to methanol. Agric Biol Chem 41 (2):423–424

Shimizu S, Ishida M, Kato N, Tani Y, Ogata K (1977b) Derepression of FAD pyrophosphorylase and flavin changes during growth of *Kloeckera* sp. No. 2201 on methanol. Agric Biol Chem 41 (II):2215–2220

Tani Y, Miya T, Nishikawa H, Ogata K (1972a) The microbial metabolism of methanol. Part I. Properties of crystalline alcohol oxidase from *Kloeckera* sp. No. 2201. Agr Biol Chem 36:68–75

Tani Y, Miya T, Ogata K (1972b) The microbial metabolism of methanol. Part II. Properties of crystalline alcohol oxidase from *Kloeckera* sp. No. 2201. Agr Biol Chem 36:76–83

Veenhuis M, Dijken JP van, Pilon SAF, Harder W (1978) Development of crystalline peroxisomes in methanol-grown cells of the yeast *Hansenula polymorpha* and its relation to environmental conditions. Arch Microbiol 117:153–156

Veenhuis M, Douma A, Harder W, Osumi M (1983) Degredation and turnover of peroxisomes in the yeast *Hansenula polymorpha* induced by selective inactivation of peroxisomal enzymes. Arch Microbiol 134:193–203

Veldkamp H, Venema PAA, Harder W, Konings WN (1966) Production of Riboflavin by *Arthrobacter globiformis*. J Appl Bact 29 (1):107–113

Wilson AC, Pardee AB (1962) Regulation of flavin synthesis by *Escherichia coli*. J Gen Microbiol 28:283–303

Received January 9, 1986/Accepted February 27, 1986